Dissection of CDK4-Binding and Transactivation Activities of p34^{SEI-1} and Comparison between Functions of p34^{SEI-1} and p16^{INK4A}†

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ABSTRACT: Recent studies showed that p34^{SEI-1}, also known as TRIP-Br1 or SEI-1, plays a dual role in the regulation of cell-cycle progression. It exhibits the transactivation activity and regulates a number of genes required for G1/S transition, while it also binds and activates cyclin-dependent kinase 4 (CDK4) independent of the inhibitory activity of p16. The goals of this paper are to further dissect the two roles and to compare the functions between SEI-1 and p16. (i) Yeast one-hybrid-based random mutagenesis was first used to identify a number of SEI-1 residues important for LexA-mediated transactivation, including residues L51, K52, L53, H54, L57, and L69 located within the heptad repeat (residues 30−88), a domain required for LexA-mediated transactivation, and two residues M219 and L228 at the C-terminal segment that contributes to transactivation through modulating the heptad repeat. (ii) The functional significance of these residues was further confirmed by site-directed mutagenesis. It was also shown that the heptad repeat-involving transactivation is distinct from the well-known acidic region-involving transactivation. (iii) Yeast two-hybrid-based binding analysis was made possible with the transactivation-negative SEI-1 mutants, and the results showed that some of such mutants retain full ability to bind and activate CDK4. (iv) Site-specific mutants of CDK4 were used to show that there are notable differences among SEI-1, p16, and cyclin D2 in binding to CDK4. (v) The expression levels of SEI-1 and p16 were compared in 32 tumor specimens of human squamous cell carcinomas of the head and neck. The results indicate that SEI-1 was consistently overexpressed, while p16 was consistently underexpressed. These results provide important information on the molecular mechanism of the functions of SEI-1 and on the comparison between SEI-1 and p16 at both molecular and cellular levels.

The newly defined gene product, p34^{SEI-1}, also known as TRIP-Br1[1] (hereafter abbreviated as SEI-1), has been shown to exhibit multiple biological functions (*1*−*3*): (a) SEI-1 specifically binds to multiple PHD zinc finger- and/or bromodomain-containing transcription factors, such as KRIP-1 and p300/CBP, and activates/represses a number of genes important for signal transduction and cell-cycle progression (*1*). (b) SEI-1 directly binds to DP1 and stimulates the transcriptional activity of the E2F1/DP1 complex in a manner that is regulated by Rb and E1A proteins, thus triggering genes required for the G1/S transition (*1*). (c) SEI-1 physically contacts cyclin-dependent kinase 4 (CDK4) through a domain distinct from its PHD-bromodomain-interacting region and stimulates CDK4-mdeiated phosphorylation of Rb (*2*, *3*). In the presence of tumor suppressor p16, a quaternary complex is formed between SEI-1, CDK4, cyclin D2, and p16, and the stimulation of SEI-1 renders the kinase resistant to the inhibitory effect of p16. (d) In a yeast one-hybrid system, SEI-1 turns on the *lac Z* reporter

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[1] Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; BSA, bovine serum albumin; CDK4, cyclin-denpendent kinase 4; DTT, dithiothreitol; DP-1, transcription factor DP-1; E2F1, transcription factor E2F1; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycobis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GST, glutathione-*S*-transferase; HEPES, *N*-[2-hydroxyethel]-piperazine-*N*′-[2-ethanisulfonic acid]; HPRT, hypoxanthine phosphoribosyltransferase; INK4, inhibitors of cyclin-dependent kinase 4, including p16 (INK4A), p15 (INK4B), p18 (INK4C), and p19 (INK4D); IPTG, isopropyl-*β*-D-thiogalactopyranose; ONPG, *o*-nitrophenyl-*β*-galactopyranose; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PEG, poly(ethylene glycol); pRb or Rb, human retinoblastoma susceptible gene product; RM, random mutagenesis; RT, reverse transcriptase; SCCHN, squamous cell carcinomas of the head and neck; SD, yeast synthetic dropout media; SDM, site-directed mutagenesis; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SEI-1, CDK4-associated protein selected with INK4A as bait, also known as TRIP-Br1; Tax, human T-cell leukemia virus type-1 transcription activator; TRIP-Br1, transcriptional regulator interacting with the PHD bromodomain; Y1H, yeast one-hybrid analysis; Y2H, yeast two-hybrid analysis.

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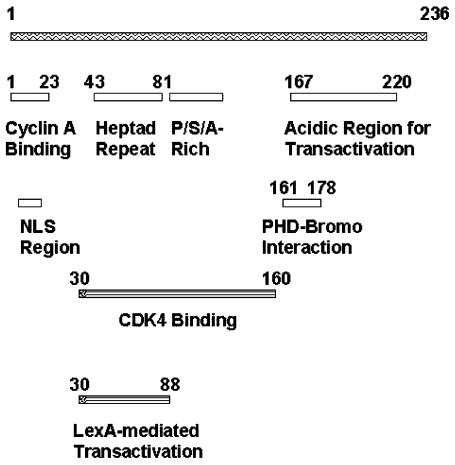

FIGURE 1: Schematic summary of the domain organization of SEI-1. This figure was modified from ref *3*. Instead of SEI-1 30−132 in ref *3*, SEI-1 30−160 was listed here as the domain responsible for binding to CDK4 because SEI-1 30−160 acts more like full-length SEI-1 in binding and modulating CDK4. NLS, nuclear localization sequence; P/S/A, praline-, serine-, and alanine-rich domain.

gene that is under the control of the LexA operator and the minimal TATA region from the *GAL1* promoter (*3*). (e) While overexpression of *SEI-1* in human cells leads to cell transformation (*2*), amplification of the *SEI-1* gene has been found in human ovarian and pancreatic tumors (*4*−*7*), suggesting that SEI-1 could play an important role in tumorigenesis.

While several functional subdomains in SEI-1 have been identified from previous studies (*1*−*3*), including the LexA-mediated transactivation subdomain in the heptad repeat (residues 30−88), the CDK4-binding region (residues 30−160), the PHD-bromodomain-interacting region (residues 167−178), the acidic region for transactivation (residues 167−217), and the C-terminal segment (residues 223−236), which is inhibitory to acidic-region-mediated transactivation (Figure 1), the specific residues important for each function, the coordination between different subdomains, and the structural bases of SEI-1 functioning remain to be determined.

In this study, we first identified a group of residues important for the LexA-mediated transactivation activity of SEI-1 through yeast one-hybrid-based random mutagenesis studies. While most of these residues are located within the heptad repeat and directly involved in LexA-mediated transactivation, two residues at the C-terminal segment contribute to transactivation through modulating the heptad repeat. We also demonstrated that some transactivation-negative SEI-1 mutants are competent in binding to CDK4 and that the binding strength of SEI-1 to CDK4 is comparable to that of cyclin D2 or p18. However, the mode of binding to CDK4 appears to be different between SEI-1, p16, and cyclin D2, as suggested by studies on a set of CDK4 mutants. Our results also suggest that SEI-1 retains two distinct transactivation activities involving the heptad repeat and the acidic region, respectively, and that binding of SEI-1 to CDK4 is independent of the heptad repeat involving transactivation. Finally, we evaluated the expression of *SEI-1* in 32 human SCCHN tumor specimens using real-time PCR, and all examined SCCHN tumor specimens demonstrated overexpression of *SEI-1* from 2- to 23-fold, while *p16* was either not expressed or was expressed at low levels in most of these tumor specimens.

MATERIALS AND METHODS

PCR-Mediated Random Mutagenesis. A Diversify PCR random muagenesis kit (BD Clontech) was used to generate a library as recommended by the manufacturer. Briefly, the PCR reaction mixture contained 5 ng of pGEX-*SEI-1* plasmid (*3*) as template, 1× TITANIUM Taq buffer, 0.64 mM $MnSO_4$, 0.2 mM dGTP, 1× Diversify dNTP mix, 0.2 μM each primer (forward and reverse), and 1 μL of TITANIUM Taq polymerse (5 units/μL) in a total volume of 50 μL. The PCR reaction included 94 °C for 30 s (1 cycle), 30 s at 94 °C and 1 min at 68 °C (25 cycles), and 1 min at 68 °C (1 cycle).

Subsequently, 1 μL of *Dpn* I (NEB product) was added into the PCR reaction mixture to digest pGEX-*SEI-1* template, and after incubation at 37 °C for 1 h, the PCR products were purified using a PCR Product Purification kit (Qiagen). Then, the PCR products were digested with both *EcoR* I and *Xho* I restriction enzymes (NEB products) and cloned into an *EcoR* I/*Xho* I-digested, dephosphorylated linear pLexA vector (BD Clontech) through cohesive end DNA ligation using T4 DNA ligase (NEB product). Finally, the ligation products were transformed into *Escherichia coli* XL-1 Blue (Stratagene), and all pLexA-*SEI-1* plasmids were amplified through mini preparation using a plasmid purification kit (Qiagen). Evidently, *SEI-1* genes in these pLexA-*SEI-1* plasmids were randomly mutated.

Yeast One-Hybrid-Based Library Screening. A yeast one-hybrid transactivation assay was used to screen the above random mutagenesis library for those SEI-1 mutants with reduced transactivation activities (*3*, *8*). pLexA-*SEI-1* plasmids were transformed into yeast strain EGY48 (BD Clontech) harboring p8op-*lacZ*, a reporter plasmid carrying the *lac Z* reporter gene under the control of the LexA operator and the minimal TATA region from the *GAL1* promoter, and transformants were selected using SD-Ura-His selective media (BD Clontech) as previously described (*8*). Replica plates were made on SD-Ura-His media containing 80 mg/L X-gal, 2% galactose, and 1% raffinose, and after incubation at 30 °C for 48 h, colonies with white and light blue colors were further evaluated using a liquid β-galactosidase enzymatic activity assay in which ONPG (Sigma) was used as the substrate. While pLexA-*CDK4* was used as negative control, pLexA-*SEI-1* wild type was used as positive control. Liquid β-galactosidase enzymatic activity assays were performed in triplicate.

For colonies with less than 50% of β-galactosidase activity of the colony containing *SEI-1* wild type, pLexA-*SEI-1* plasmids were recovered as previously described (*9*) and subsequently sequenced using an ABI377A automated DNA sequencer. Both strands of pLexA-*SEI-1* plasmids were sequenced to confirm the mutations. Of note, the above molecular-cloning procedure needs to be optimized to increase the efficiency of the random mutagenic library. For example, instead of transforming and amplifying the ligation products into bacteria prior to lithium acetate/PEG-mediated yeast transformation, directly transforming the ligation products into yeast through electroporation will be more efficient (*9*).

To single out the role of each mutation in transactivation, single, double, and triple *SEI-1* mutants were generated through PCR-mediated Quickchange mutagenesis (Stratagene) using pLexA-*SEI-1* wild type as the template and their transactivation activities were evaluated as above.

Yeast Two-Hybrid Analysis of Interactions between CDK4 and Partner Proteins Including SEI-1, INK4, and Cyclin D2. To evaluate the interaction between transactivation-negative SEI-1 mutants and CDK4, human *CDK4* cDNA was cloned into the activation domain fusion vector, pB42AD (BD Clontech), and cotransformed into EGY48/p8op-*LacZ* with pLexA-*SEI-1* plasmids. The binding of SEI-1 mutants to CDK4 was represented by the activation of the *lac Z* gene, and the binding affinity was quantitatively evaluated in liquid β-galactosidase activity assays as previously described (*9*).

For the domain-switching experiments (*9*), genes encoding transactivation-negative SEI-1 mutants were cloned into pB42AD as activation domain fusion plasmids and human *CDK4* cDNA was cloned into pLexA as a binding domain fusion plasmid (*10*). Similarly, pB42AD-*p16*, pB42AD-*p18*, and pB42AD-*cyclin D2* plasmids (*10*) were cotransformed with pLexA-*CDK4* to evaluate the CDK4-binding ability of p16, p18, and cyclin D2, respectively.

CDK4 mutants K22Q, R24C, and N41S were generated through PCR-mediated Quickchange mutagenesis (Stratagene) using pLexA-*CDK4* wild type as the template.

Cloning, Expression, and Purification of SEI-1 Wild-Type and Mutant Proteins. As previously described (*3*), cDNA genes encoding SEI-1 wild-type and mutant proteins were cloned into pGEX-6p-1 vectors (Amersham Pharmacia) at *EcoR* I/*Xho* I sites and expressed in *E. coli* BL21 (DE3) Codon plus cells (Novagene) as glutathione-*S*-transferase (GST) fusion proteins upon IPTG induction. GST-fusion proteins were purified from the cell lysate using a reduced glutathione-agarose column (Sigma) followed by a Q Fast-flow column (Pharmacia). To purify free SEI-1 proteins, 100 units of PreScission protease (Amersham) were added to GST-SEI-1 proteins in PBS buffer at the final concentration of 2 units/mL. After incubation at 4 °C for 24 h, the reaction mixture was loaded onto a PBS-equilibrated reduced glutathione-agarose column to remove GST and the flow through was collected and concentrated. The final product was analyzed by SDS−PAGE, and its purity was about 90%.

In Vitro CDK4 Kinase Assay. The *in vitro* CDK4 activity assay was performed as previously described (*11*). Each reaction mixture contained about 0.15 μg of recombinant CDK4/cyclin D2 holoenzyme and varying concentrations of SEI-1 proteins in 15 μL of the kinase buffer, 50 mM HEPES, 10 mM $MgCl_2$, 2.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM NaF, 10 mM β-glycerolphosphate, 1 mM DTT, 0.2 mM AEBSF, 2.5 mg/mL leupeptin, and 2.5 mg/mL aprotinin. After incubation at 30 °C for 30 min, 100 ng of GST-Rb791−928 and 5 μCi [γ-^{32}P]ATP were added in the reaction mixture and the reaction mixture was incubated at 30 °C for another 15 min. Proteins in the reaction mixture were separated by SDS−PAGE, and the incorporation of ^{32}P into GST-Rb791−928 was quantitatively evaluated using a PhosphorImager (Molecular Dynamics). Measurements were repeated in triplicate.

Recombinant CDK4/cyclin D2 holoenzyme was expressed and purified as follows (*11*). Human *CDK4* and *cyclin D2* cDNAs were cloned into pBacBAK8 and pBacBAK6 vectors (BD Clontech), respectively. Of note, a 6× His tag was fused to the C terminus of CDK4 to facilitate the following purification of the CDK4/cyclin D2 holoenzyme. Each construct was cotransfected into *Spodoptera frugiperda* SF-9 cells with *Autographa* California nuclear polyiruhedrosis virus *BacPAK6/Bsu-361* DNA (BD Clontech) to generate baculovirus particles. Both baculovirus particles were cotransfected into HighFive insect cells (Invitrogen), and the CDK4/cyclin D2 holoenzyme was purified through affinity chromatography using Talon resin (BD Clontech). The final product was concentrated to approximately 0.3 mg/mL in the above kinase buffer, and aliquots were stored at −80 °C.

The substrate of CDK4, GST-Rb791−928, was expressed and purified from bacteria as described above using a pGEX-2T vector (Amersham) in which cDNA-encoding human Rb residues 791−928 was fused to the 3′ end of *GST* in the open-reading frame (*12*).

Sample Procurement and Total RNA Extraction. Specimens were obtained from the Tissue Procurement Service at the Ohio State University Comprehensive Cancer Center as previously described (*13*). Specimens were either quickly frozen in liquid nitrogen in individual plastic bags following surgical resection or placed in RNA-later solution (Ambion) immediately following excision. After the frozen tissue was crushed by repetitive hammering, it was dissolved in TRIzol extraction reagent (Invitrogen) and total RNA extraction was performed according to the instructions of the manufacturer. RNA concentration and purity were determined spectrophotometrically by absorbance at 260 and 280 nm. RNA was aliquoted and stored at −80 °C, and each aliquot was thawed only once and used immediately.

First-Strand cDNA Synthesis. The first-strand cDNA synthesis was performed using PowerScript Reverse Transcriptase (BD Clontech) as follows: a reaction mixture containing 2.0 μL of total RNA (∼2−5 μg), 2.0 μL of 5× PowerScript RT buffer, 1.0 μL of Oligo $d(T)_{12-18}$ (0.5 μg/μL), and 1.0 μL of Random Hexamers (50 μM) was incubated at 70 °C for 7.5 min and then chilled to 4 °C for 10 min. Subsequently, 2.0 μL of 5× PowerScript RT buffer, 2 μL of DTT (0.1 M), 1 μL of dNTPs mixture (25 mM each individual dNTP), 2 μL of 5× PowerScript RT, and 3 μL of distilled water were combined into the reaction mixture, and the reaction mixture was incubated at the following conditions: 30 °C for 5 min, 37 °C for 30 min, 42 °C for 50 min, 50 °C for 5 min, and 80 °C for 10 min. After incubation with 1 μL of RNase H (Amersham Biosciences) at 37 °C for another 30 min, the reaction mixture was chilled to 4 °C for 10 min and stored on ice for use.

Real-Time PCR Amplification. Amplification reactions were carried out using the Cepheid SmartCycler system (Cepheid), and primers and probes were designed using Primer Express Software version 2.0 (Applied Biosystems). Each reaction was performed in the presence of 200 μM dNTPs, 3 mM $MgSO_4$, 1.25 units of Taq polymerase (Stratagene), 1× Additive (1 mg/mL BSA, 750 mM Trehalose, and 1% Tween 20), 2.0 μL of first-strand cDNA (containing about 0.5−1.0 μg of cDNA), 0.5 μL of each primer (at a final concentration of 0.2 μM), and 0.25 μL of the dual-labeled fluorogenic probe (at the final concentration of 0.1 μM). The sample was subjected to 96 °C for 2 min, followed by 55 cycles of 30 s at 95 °C, 30 s at 60 °C, and

30 s at 68 °C. A final extension step was performed at 68 °C for 5 min. A housekeeping gene, human hypoxanthine phosphoribosyltransferase (*HPRT*) was used as the reference because it was consistently and reproducibly expressed in all samples. The primers and fluorogenic probes used in amplifying different cDNA genes were for *SEI-1*, 5′-GGCCTCTAGCTCCCTCTCCGA-3′ (forward), 5′-AGGTCCGGCTCACTCTGCT-3′ (reverse), and 5′-FAM-TCTCAGTGCTCAAGCTCCACCACAGC-TAMRA-3′ (probe); for *p16*, 5′-TGCCCAACGCACCGA-3′ (forward), 5′-GGGCGCTGCCCATCA-3′ (reverse), and 5′-TET-TAGTTACGGTCGGAGGCCGATCCA-TAMRA-3′ (probe); and for *HPRT*, 5′-CGGCTCCGTTATGGCG-3′ (forward), 5′-GGTCATAACCTGGTTCATCATCAC-3′ (reverse), and 5′-FAM-CGCAGCCCTGGCGTCGTGA-TAMRA-3′ (probe). Each gene was amplified separately, and all experiments were performed in duplicate.

Gene Expression Analysis. The accuracy of the above real-time PCR assays was verified using serial dilutions of plasmid DNAs containing human *SEI-1*, *p16*, and *HPRT* cDNA genes, respectively. The plasmid DNA concentration was spectrophotometrically determined by absorbance at 260 nm, and after a serial 10-fold dilution, plasmid DNA samples were analyzed using the above real-time PCR assays. The resulting cycle threshold (Ct) values were plotted against the amount of plasmid DNA (in exponential form). A linear graph with a correlation coefficient of 0.99 is the indicator showing that the concentration of the target cDNA gene can be accurately measured using the above assays, thus justifying the above assays (*14*).

The concentrations of *SEI-1*, *p16*, and *HPRT* genes in each sample were derived from the threshold cycle (Ct) values of corresponding genes using the above standard curves (Ct versus the concentration in exponential form) and were normalized to get the relative gene expression using the following formula (*15*):

$$\text{relative expression of target gene} = (\text{concentration of target gene}/\text{concentration of } HPRT)_{\text{tumor}} \div (\text{concentration of target gene}/\text{concentration of } HPRT)_{\text{control}}$$

RESULTS

Random Mutagenesis. Our previous studies have demonstrated that SEI-1 functions in both CDK4 activation and LexA-mediated transactivation (*3*). However, our previous results did not dissect the CDK4-binding region (residues 30−160) from the heptad repeat of SEI-1 (residues 30−88) possessing the transactivation activity, nor did we identify specific residues responsible for these activities. To further identify those residues important for LexA-mediated transactivation, we constructed a library of SEI-1 mutants through PCR-mediated random mutagenesis followed by a yeast one-hybrid-based functional screening (*8*). Because other domains could potentially contribute to the transactivation activity of the heptad repeat, the gene encoding the intact SEI-1, not just the heptad repeat domain, was used as the template. After color development, 31 colonies in light blue and white were screened out of about 5200 yeast colonies. Further quantitative evaluation of the LexA-mediated transactivation activity indicated that 19 colonies exhibited less than 50% of the transactivation activity of SEI-1 wild type. pLexA-*SEI-1* plasmids were recovered from these 19 colonies for DNA sequencing, and 11 SEI-1 mutants with reduced transactivation activities were identified (Table 1).

Table 1: Relative LexA-Mediated Transactivation Activities of SEI-1 Mutants from Random Mutagenesis

mutant ID[a]	mutations	relative transactivation activity[b] (%)
	SEI-1 wild type	100
	LAM	0
RM01	M219I	29.4
RM02	H54L, L69P, N72S, L173Q, T179A	4.9
RM08	L157P, D211G, L228P	4.1
RM09	L228P	4.3
RM10	L53P, T73A, L157P	12.7
RM11	H54L, E138G, N184S	9.9
RM15	L53P, M115V, H124R, L166P, E171G	2.4
RM22	L57P, S117R	13.2
RM23	L51P, L119P, L122P, Y182H, D216A	12.2
RM24	S49STOP, L119P, L157P	0
RM29	L51P, L53H, L69P, D108G, Y182N	0

[a] LAM is a transactivation-negative control provided by the manufacturer (BD Clontech). [b] Transactivation assays were performed in triplicate, and the relative activity was defined as 100 × the mean value of a mutant/the mean value of the wild type.

As can be seen in Table 1, all 11 mutants from random mutagenesis exhibited significantly reduced LexA-mediated transactivation activities and 2 of them, RM24 and RM29, did not exhibit any detectable LexA-mediated transactivation activities. While RM24 contains a Ser → stop mutation at residue 49, RM29 is a transactivation-negative mutant of great interest. There are 5 mutations at RM29, including L51P, L53H, L69P, D108G, and Y182N, suggesting that some or all of these residues may play important roles in LexA-mediated transactivation. Moreover, 8 of these 11 mutants contain mutations at residues located in the heptad repeat (residues 30−88), a domain important for LexA-mediated transactivation as demonstrated in our previous studies (*3*). The remaining 3 mutants contain mutations at the C-terminal segment, including single mutants M219I and L228P and a triple mutant L157P/D211G/L228P, suggesting that, besides the heptad repeat, the C-terminal segment of SEI-1 also contributes considerably to the LexA-mediated transactivation.

Site-Directed Mutagenesis: Residues Important for Transactivation. To further narrow down those residues important for LexA-mediated transactivation, we designed a series of single, double, and triple mutants based on the following reasoning. First, for random mutants containing mutations in the heptad repeat, single, double, or triple mutants in the heptad repeat residues were first generated and evaluated. If mutants in the heptad repeat residues did not cause changes in transactivation comparable to those found in corresponding random mutants, then residues out of the heptad repeat were mutated. Second, because mutant RM29, L51P/L53H/L69P/D108G/Y182N, has lost all of its transactivation activity, single mutants at all five residues were designed to evaluate the contribution from each residue to LexA-mediated transactivation. Subsequently, double and triple mutants were generated to evaluate the potential additive effect among RM29-related single mutants, especially those Leu mutants

Table 2: Relative LexA-Mediated Transactivation Activities of SEI-1 Mutants from Site-Directed Mutagenesis

mutant ID[a]	mutations	relative transactivation activity[b] (%)	mutant ID	mutations	relative transactivation activity (%)
	SEI-1 wild type	100	SDM09	L57A	2.62
	LAM	0	SDM10	L57P	1.12
	SEI-1 30−236	104.5	SDM11	L69A	1.19
	SEI-1 30−160	67.2	SDM12	L69P	0.34
	SEI-1 30−88	62.8	SDM13	N72A	93.4
	SEI-1 89−236	0	SDM14	N72S	114.2
			SDM15	T73A	108.4
SDM01	L51A	33.6	SDM16	D108G	89.3
SDM02	L51P	14.1	SDM17	Y182N	95.1
SDM03	K52A	1.9	SDM18	L228A	5.8
SDM04	L53A	1.23	SDM19	L51A, L69A	0
SDM05	L53H	0.82	SDM20	L53H, L69A	0
SDM06	L53P	0.71	SDM21	L53H, L228A	0.74
SDM07	H54A	0.53	SDM22	L69A, L228A	0.98
SDM08	H54L	1.49	SDM23	L51A, L53H, L69A	0

[a] LAM is a transactivation-negative control provided by the manufacturer (BD Clontech). [b] Transactivation assays were performed in triplicate, and the relative activity was defined as 100 × the mean value of a mutant/the mean value of the wild type.

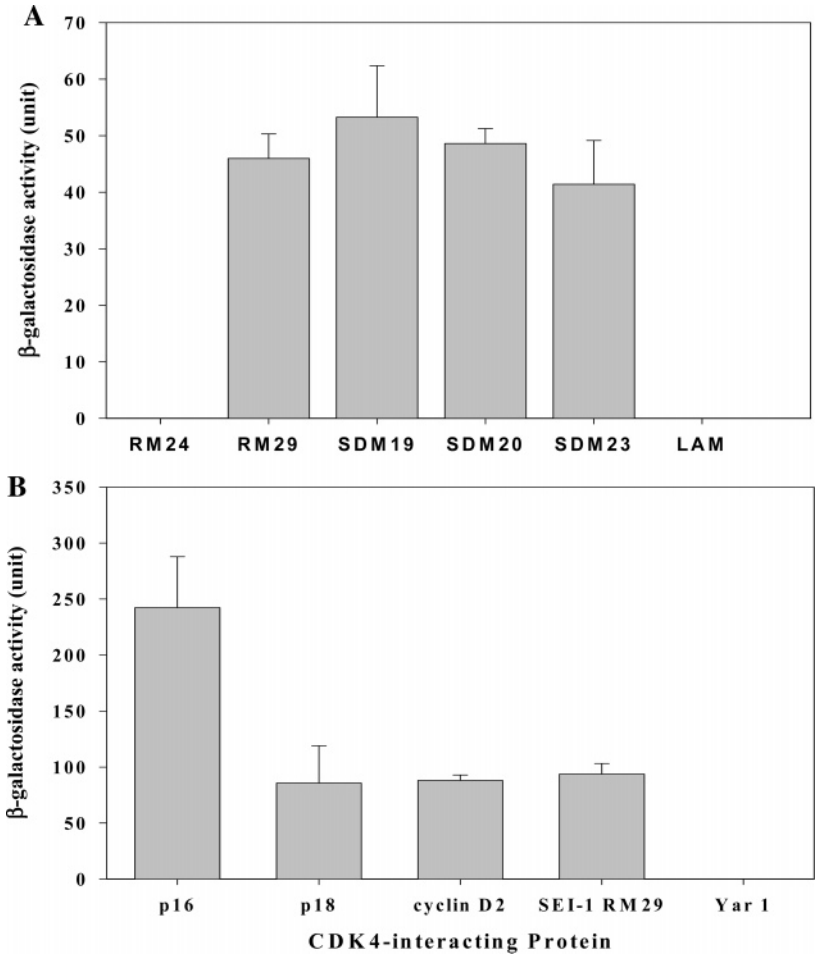

FIGURE 2: Yeast two-hybrid analyses to evaluate interactions between CDK4 and SEI-1 mutants that lack LexA-mediated transactivation activity. (A) Interactions between CDK4 and SEI-1 proteins. In this assay, pLexA-*SEI-1* plasmids were cotransformed into EGY48/p8op-*LacZ* with pB42AD-*CDK4*. *β*-Galactosidase activities from transformants with both pLexA-*SEI-1* and pB42AD-*CDK4* plasmids represent the CDK4-binding strength of SEI-1 mutants. LAM is a negative control provided by the manufacturer (BD Clontech). (B) Interactions between CDK4 and its partner proteins. In this assay, the *CDK4* gene was cloned into the binding domain fusion vector pLexA, and *p16*, *p18*, *cyclin D2*, and *SEI-1 RM29* genes were cloned into the activation domain fusion vector pB42AD. Yar-1, a yeast ribosomal protein without any interactions with CDK4 was used as a negative control as previously described (*10*, *12*). In both A and B, measurements were repeated in triplicate and error bars represent standard deviations.

that are involved in the formation of the leucine zipper in the heptad repeat (*1*). Third, double mutants including L228A and residues at the heptad repeat were generated to evaluate the coordination effect between the heptad repeat and the C-terminal segment that is also involved in LexA-mediated transactivation (Table 1). Fourth, the potential role of K52 in LexA-mediated transactivation was also evaluated because mutations at the neighboring residues including L51, L53,

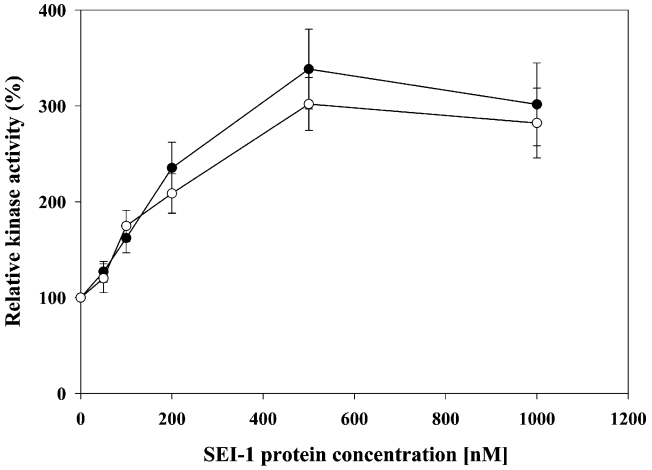

FIGURE 3: *In vitro* kinase assay to evaluate SEI-1-mediated activation of CDK4. Each reaction mixture included 1.5 units of CDK4-cyclin D2 holoenzyme (about 0.15 μg of protein), 100 ng of GST-Rb791−928, 5 μCi of [γ-^{32}P]ATP, and a varying amount of SEI-1 proteins. After incubation at 30 °C for 15 min, phosphorylated GST-Rb791−928 was separated by SDS−PAGE, and the incorporation of ^{32}P into Rb was quantified with a PhosphorImager. SEI-1 wild type, ●; SEI-1 RM29, ○. All measurements were repeated in triplicate, and error bars represent standard deviations. The kinase activity in the absence of SEI-1 proteins is used as the reference point at 100%.

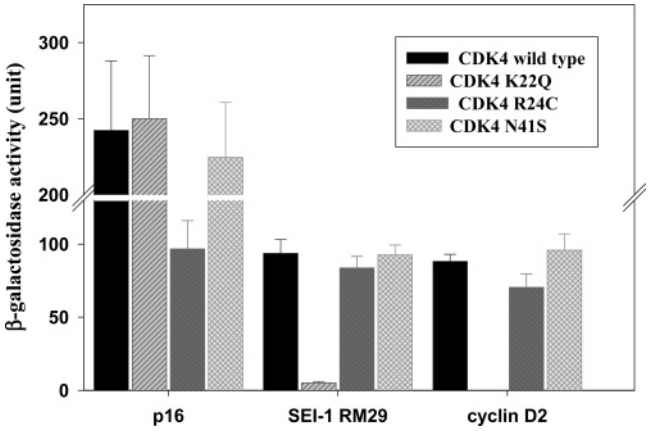

FIGURE 4: Yeast two-hybrid analyses for determining the interactions between CDK4 mutants and partner proteins including p16, SEI-1 RM29, and cyclin D2. Experiments were performed as described in the caption of Figure 2B, and error bars represent standard deviations.

and H54 have been found in the random mutagenesis experiments. Table 2 summarizes the results of yeast one-hybrid-based transactivation assays for all of the site-specific mutants.

For the residues within the haptad repeat, mutants at N72 and T73 behaved as wild-type SEI-1 and L51A and L51P exhibited modest decreases (3- and 7-fold reductions, respectively) in LexA-mediated transactivation activity. However, mutations of K52, L53, H54, L57, and L69 all led to a significant loss of LexA-mediated transactivation activity (retaining less than 5% of the activity of SEI-1 wild type), suggesting that these residues play important roles in LexA-mediated transactivation.

For the residues outside of the haptad repeat, D108G and Y182N mutants did not show notable changes in the LexA-mediated transactivation activity after mutation, suggesting that these two residues are not involved in LexA-mediated transactivation. This is consistent with the following observations. First, our previous fragmentation studies have demonstrated that the segment encompassing residues 88−160 is responsible for CDK4 binding and stimulation (*3*). Second, similar to RM29 (L51P/L53H/L69P/D108G/Y182N), the triple mutant L51A/L53H/L69A is also transactivation-negative.

Then, we evaluated the additive effect. While L51A, L53H, and L69A retained 33.6, 0.82, and 1.19% of the LexA-mediated transactivation activity of SEI-1 wild type, respectively, L51A/L69A, L53H/L69A, and L51A/L53H/L69A did not exhibit any detectable LexA-mediated transactivation activity. In contrast, while single mutant L228A retains 5.8% of the transactivation activity of SEI-1 wild type, the LexA-mediated transactivation activities of L53H/L228A (0.74%) and L69A/L228A (0.98%) were almost identical to those of single mutants L53H (0.82%) and L69A (1.19%), respectively. Thus, there is no apparent additive effect between mutations at the heptad repeat and the C-terminal segment.

Some Transactivation-Negative SEI-1 Mutants Can Bind to and Activate CDK4. Binding of SEI-1 to CDK4 has been demonstrated in pull-down assays (*2*, *3*); however, quantitative evaluation of this interaction was impeded because of the following reasons. First, it is challenging to get a large amount of pure SEI-1 for any *in vitro* kinetic studies. Second, the transactivation activity of SEI-1 complicates the yeast two-hybrid-based measurement of binding affinity (*3*). Identification of transactivation-negative SEI-1 mutants enabled us to evaluate the potential interactions between CDK4 and these SEI-1 mutants through yeast two-hybrid analyses. In this study, binding domain fusion vectors pLexA-*SEI-1* mutants (including RM24, RM29, L51A/L69A, L53H/L69A, and L51A/L53H/L69A) were cotransformed into yeast strain EGY48/p8op-*Lac* Z with an activation domain fusion plasmid, pB42AD-*CDK4*. For a colony containing both pLexA-*SEI-1* mutant(s) and pB42AD-*CDK4*, β-galactosidase activity represents the strength of the CDK4/SEI-1 mutant interaction. As shown in Figure 2A, RM24, the deletion mutant with S49STOP mutation did not show any CDK4-binding activity. However, RM29 and three site-specific mutants SDM 19, 20, and 23, all of which were transactivation-negative, exhibited considerable and comparable CDK4-binding activities. Therefore, we chose RM29 as a representative mutant for further analyses because RM29 contains 5 mutations and it is the best mutant to show that CDK4 binding of SEI-1 is independent of LexA-mediated transactivation.

The interaction between RM29 and CDK4 was further evaluated in the Y2H system through domain switching (*9*). In this assay, the gene encoding RM29 was cloned into the activation domain fusion vector as pB42AD-*RM29*, while *CDK4* cDNA gene was cloned into the binding domain vector as pLexA-*CDK4*. As shown in Figure 2B, RM29 interacted with CDK4, suggesting that the RM29/CDK4 interaction observed in the Y2H system is not an artifact. Furthermore, we assessed the potential of RM29 in stimulating CDK4 using an *in vitro* kinase assay (*3*, *11*). As shown in Figure 3, the CDK4 kinase activity increased with increasing concentrations of RM29 and the stimulating potential of RM29 was comparable to that of SEI-1 wild type. When these results are taken together, they demonstrate

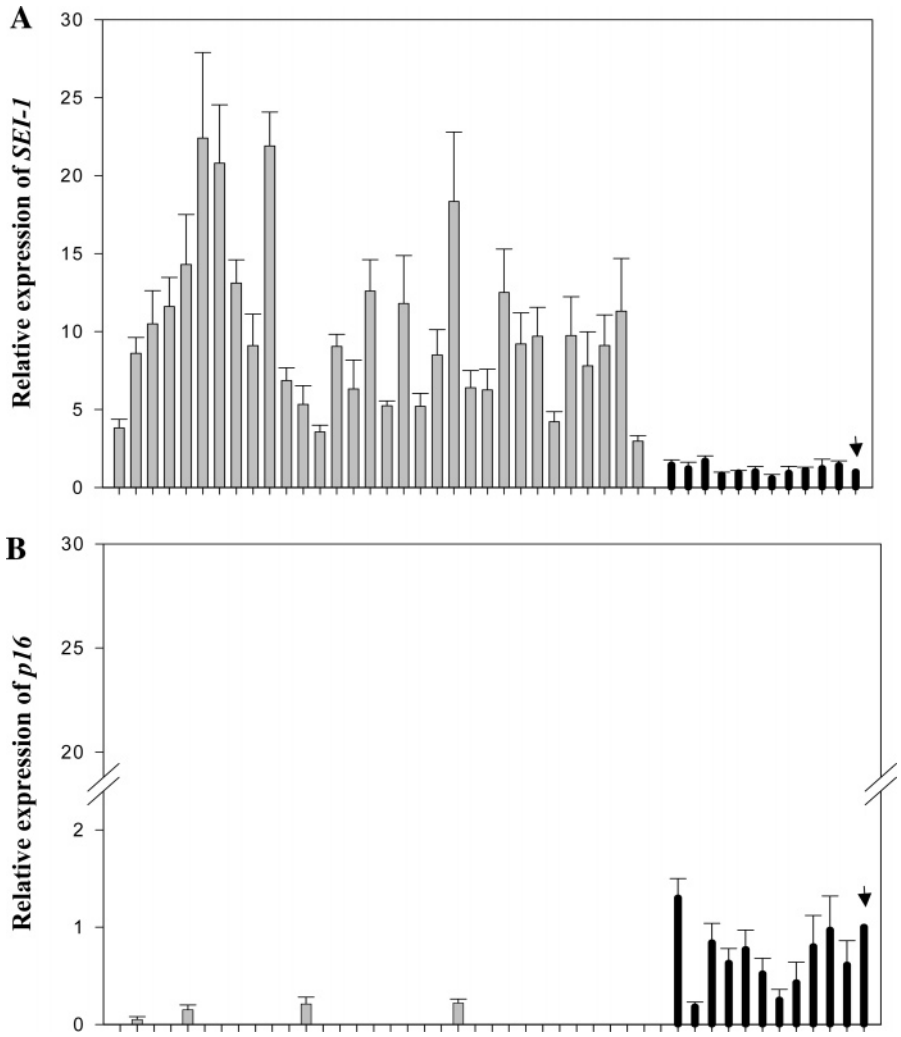

FIGURE 5: Real-time PCR assays to evaluate the expression of SEI-1 (A) and p16 (B) in human squamous cell carcinomas of the head and neck (SCCHN) and normal tissues. A total of 32 specimens from SCCHN (in gray) and 11 specimens from corresponding normal tissues (in black) were analyzed using real-time PCR, and a pooled sample (↓) from these 11 normal specimens was used as a control with a relative expression value of 1. All experiments were performed in duplicate, and error bars represent standard errors.

that LexA-mediated transactivation-negative RM29 retains full ability to bind and activate CDK4, indicating that LexA-mediated transactivation and CDK4 binding/activation are independent of each other.

In addition, we compared SEI-1 and other CDK4 partner proteins in binding strength using yeast two-hybrid analyses (*10*, *12*). As shown in Figure 2B, the binding strength of SEI-1 to CDK4 is weaker than that of p16 but comparable to that of p18 or cyclin D2. While the signal strength in the yeast two-hybrid assay may not always correlate well with the actual binding affinity of protein/protein interactions in cells, these results suggest that, from the perspective of binding affinity, the effect of SEI-1 on CDK4 could be regarded as comparable to those of other CDK4 partners under certain conditions.

Use of CDK4 Mutants to Compare the Binding Modes between SEI-1 and p16. We also investigated the interactions of SEI-1 (the R29M mutant) and three CDK4 mutants K22Q, R24C, and N41S, which have been found in human cancers (*16*). The purpose was to compare such interactions with p16 and cyclin D2, which have already been established previously. K22 of CDK4 is known to be involved in binding to cyclin Ds, and K22Q exhibits a significant decrease in CDK4 kinase activity but retains p16-binding ability (*12*, *16*, *17*). R24C mutation has been shown to abolish the CDK4 inhibitory activity of p16 and to cause a modest decrease in its binding affinity (*12*, *18*), likely because of disruption of an electrostatic interaction between R24 of CDK4 and D84 of p16 as revealed in the crystal structures of p16/CDK6 and p19/CDK6 complexes (*19*, *20*). The role of N41S of CDK4 is not clear, and this mutation does not affect p16 binding (*12*, *16*). As shown in Figure 4, CDK4 wild type and the three mutants show different patterns of interaction with p16, the RM29 mutant of SEI-1, and cyclin D2. Mutation at R24 of CDK4 did not result in any detectable change in binding to SEI-1, which is consistent with the previous studies showing that SEI-1 does not compete with p16 for binding to CDK4 but forms a quaternary complex with CDK4, cyclin D2, and p16 (*2*, *3*). Interestingly, the K22Q mutation abolished its cyclin D2-binding ability and also led to a loss of more than 90% of SEI-1-binding ability. Seemingly, this is not well-consistent with our previous observation that binding of SEI-1 to CDK4 does not disrupt the association between CDK4 and cyclin D2 (*3*). While the molecular basis of the effect of the K22Q mutation on the binding of SEI-1 requires further investigation, one possibility is that K22 of CDK4, a residue required for cyclin D2 binding, is not directly involved in binding to SEI-1, but

K22Q mutation may bring about a local conformational change that renders CDK4 unfavorable for SEI-1 binding. When these observations are taken together, they indicate that there are notable differences in the molecular basis of CDK4 binding to SEI-1, p16, and cyclin D2. Structural studies of CDK4−SEI-1 interactions will thus be of great interest.

Comparison of SEI-1 and p16 Expressions in Human SCCHN Tumor Specimens. The human *SEI-1* gene is located at chromosome 19q13.1−13.2 (*4*). This locus contains another oncogene, *Akt2*, and has been found to be amplified in a number of human tumors (*4*−*7*). It has been also reported that expression of *SEI-1* in human cancer cell lines was induced by the addition of serum in cell cultures (*2*). These features suggest that the expression of *SEI-1* could contribute to the development of human cancers. In this study, we developed assays to accurately determine the expression of *SEI-1*, *p16*, and hypoxanthine phosphoribosyltransferase (*HPRT*, as a control) in human SCCHN tumor specimens using real-time quantitative fluorescence PCR. Evaluation of plasmids containing *SEI-1*, *p16*, and *HPRT* cDNA genes with known concentrations confirmed that these assays have a dynamic range wide enough for clinical samples (data not shown). For real-time PCR of each gene, when plotted exponentially, the Ct values correlated linearly ($r^2 = 0.99$) with known amount of plasmids containing the target gene (data not shown), indicating validity of these assays (*14*). Because *HPRT* is a housekeeping gene consistently and reproducibly expressed in all specimens, it was used as an internal control. The expression level of *SEI-1* or *p16* in a specimen was defined as the ratio of *SEI-1* or *p16* to *HPRT* (*15*) and was further normalized in comparison with the expression level of *SEI-1* or *p16* in a pooled sample of 11 normal specimens. As shown in Figure 5A, an increase of 2−23-folds in the expression of *SEI-1* was observed in all examined SCCHN tumor specimens relative to the expression of *SEI-1* in 11 normal specimens. In contrast, *p16* was either not expressed or was expressed at low levels in all of these tumor specimens (Figure 5B). While a number of studies have demonstrated that inactivation of *p16* by deletions, mutations, and methylations prevalently contribute to human SCCHN (*21*), our studies demonstrated for the first time that *SEI-1* is frequently overexpressed in human SCCHN tumor specimens.

DISCUSSION

Potential Importance in Examining Both SEI-1 and p16 in Tumor Tissues. The results described above suggest that overexpression of *SEI-1* could play an important role in the development of human SCCHN. There are three different mechanisms in which overexpression of *SEI-1* could affect the INK4−CDK4/6-Rb(E2F) pathway (*1*, *22*), including (a) stimulating CDK4-mediated phosphorylation of Rb through physical association with CDK4, (b) activating E2F-responsible promoters through the formation of the E2F1/DP-1/SEI-1 ternary complex, and (c) transactivating *cyclin E* gene and subsequently activating CDK2−cyclin E-mediated phosphorylation of Rb. Regardless, the physiological consequence is likely the same as that caused by the inactivation of *p16*, hyperphosphoryaltion of Rb and the subsequent activation of E2F-controlled promoters. The actual cellular effects remain to be verified with biological

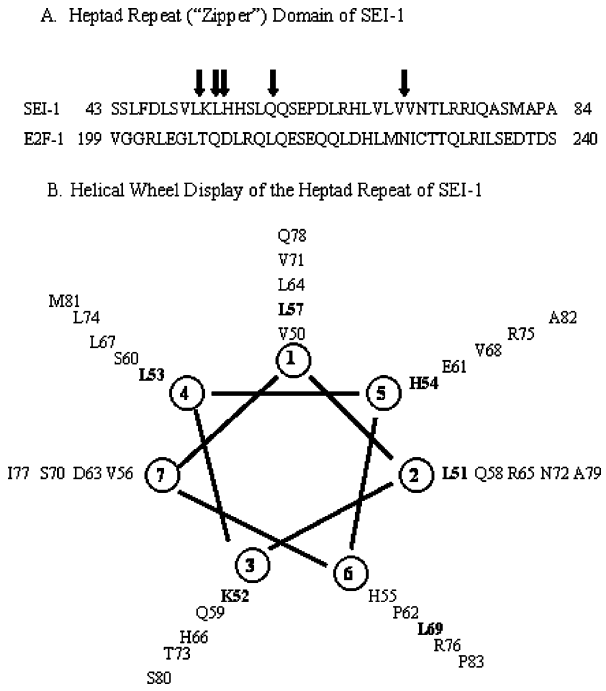

FIGURE 6: Amino acid sequence and helical wheel display of the heptad repeat of SEI-1. (A) Amino acid sequences of the heptad repeat domains in SEI-1 and E2F1. Arrows represent residues important for transactivation identified from this study. (B) Helical wheel display of the heptad repeat of SEI-1. Residues important for transactivation are highlighted. This figure is modified from ref *1*.

experiments. However, our results suggest that both overexpression of *SEI-1* and low expression of *p16* may contribute to the development of SCCHN. We believe this is an important finding, because in some cancer cells the expression level of p16 has been found to be normal or even enhanced (*13*, *23*−*27*). It is possible that *SEI-1* is significantly overexpressed in these cells. In any case, it is important to examine the expression of both proteins when investigating their roles in cancers.

SEI-1 May Retain Two Distinct Transactivation Activities. SEI-1 is a transcriptional activator that positively or negatively regulates a number of important genes, such as *cyclin E* gene (*1*, *22*). While Hsu and his colleagues have demonstrated that SEI-1 retains a transactivation activity attributed to its acidic region (residues 167−217) (*1*), our studies focus on the LexA-mediated transactivation of SEI-1 involving the heptad repeat (residues 30−88). There are notable differences between these two types of transactivations. First, our results indicate that the LexA-mediated transactivation is independent of residues in the acidic region. Mutations in the heptad repeat regions, such as mutants L51A/L69A, L53H/L69A, and L61A/L53H/L69A, are sufficient to abolish all LexA-mediated transactivation, and the truncated SEI-1 (89−236) did not exhibit any detectable LexA-mediated transactivation activity (Table 2). No single mutation in the acidic region has been shown to significantly reduce the LexA-mediated transactivation. Second, the residues C-terminal to the acidic region appear to play an inhibitory role in the acidic region-responsible transactivation but contribute positively to the LexA-mediated transactivation. Removal of residues 223−236 brought about an increase in the acidic region-responsible transactivation (*1*),

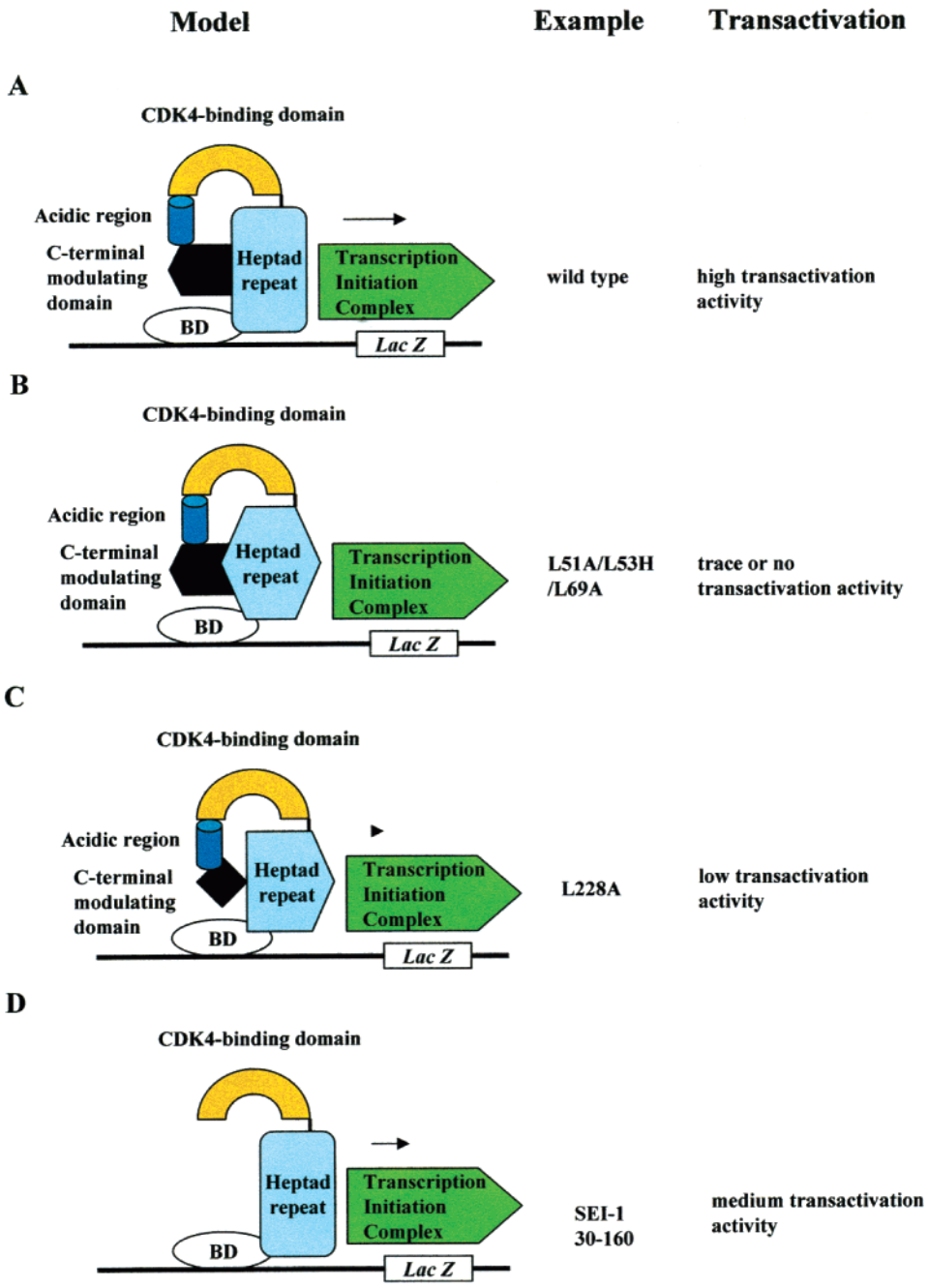

FIGURE 7: Model of LexA-mediated transactivation of SEI-1. The coordination between the heptad repeat (residues 30−88) and the C-terminal segment under different conditions is described in the text. Only four SEI-1 domains, the heptad repeat (residues 30−88, in light blue), the CDK4-binding domain (residues 89−160, in orange), the acidic region (residues 167−217, in dark cyan), and the C-terminal segment (residues 219−236, in black) are shown here. BD represents the GAL4-binding domain from the pLexA plasmid; *LacZ* is the reporter gene in the p8op-*LacZ* plasmid; and arrows indicate the transcription direction of the *lacZ* gene.

while mutation of some residues in the C-terminal region (M219I and L228A) led to moderately decreased activities of LexA-mediated transactivation (Tables 1 and 2). Third, a residue N-terminal to the heptad repeat has been shown to contribute positively to the acidic region-mediated transactivation as shown by the reduced activity of E12K (*1*), while the truncated mutant SEI-1 30−236 retains full LexA-mediated transactivation activity. The molecular mechanisms underlining the dual transactivation activities of SEI-1 as demonstrated in Hsu et al. (*1*) and our studies and the physiological significance of these dual transactivation activities of SEI-1 are not clear yet. However, it is worthwhile to note that the experimental systems used in these studies are different. In the mammalian cell system used by Hsu et al., the luciferase reporter gene was under control of the GAL4 DNA-binding sequence followed by the E1b TATA box (*1*), while in our yeast cell system, the lac Z reporter gene was regulated by the LexA operator and the minimal TATA region from the GAL1 promoter (*3*). It has been reported that SEI-1 functions to activate some genes but repress others (*1*); hence, it is very likely that SEI-1 functions differently to regulate different transcription machineries. Evidently, it will be of interest to introduce SEI-1 mutants with different transactivation activities into cells and identify genes controlled by different transactivation activities of SEI-1.

Structural Model for the LexA-mediated Transactivation. The heptad repeat of SEI-1 is a relatively conserved domain also present in transcription factor E2F1 (Figure 6A), and a helical wheel analysis shows that this domain contains a "zipper" helix starting from residue V50 (Figure 6B). In the present study, we further demonstrated that six residues in the heptad repeat, including L51, K52, L53, H54, L57, and L69, are important for LexA-mediated transactivation. All of these six residues are located within the helical region of the heptad repeat, and mutations at these residues lead to the loss of LexA-mediated transactiavtion activity. In addition to the fact that the heptad repeat itself retains considerable amount of the LexA-mediated transactivation activity of intact SEI-1, SEI-1 89−236 does not have any detectable LexA-mediated transactivation activity (Table 2), indicating that the heptad repeat is required and sufficient for LexA-mediated transactivation. Furthermore, our results have clearly dissected the regions responsible for the CDK4 binding and the LexA-mediated transactivation.

When these features are taken together, they lead us to propose a novel model for LexA-mediated transactivation (Figure 7). In this model, the C-terminal segment of SEI-1 (residues 219−236) contributes to the full activity of SEI-1 in LexA-mediated transactivation by modulating the heptad repeat, the subdomain primarily responsible for the LexA-mediated transactivation. The CDK4-binding domain and the acidic region are located between the heptad repeat and the C-terminal segment and contribute little to LexA-mediated transactivation (Figure 7A). Mutations within the heptad repeat, such as L51A/L53H/L69A, could disrupt or distort the conformation of this helix and lead to the loss of LexA-mediated transactivation activity (Figure 7B), even though the C-terminal segment is intact. Mutations within the C-terminal segment, such as L228A, could perturb the interaction between the heptad repeat and the C-terminal segment and lead to substantially reduced LexA-mediated transactivation activities (Figure 7C). In truncated mutants such as SEI-1 30−160 and SEI-1 30−88, which are devoid of the C-terminal segment, the acidic region, and/or the CDK4-binding domain, the LexA-mediated transactivation activity is only moderately reduced possibly because the heptad repeat is able to adapt a more flexible or favorable conformation in the absence of the modulating C-terminal residues (Figure 7D). This model represents only our working hypothesis, and it remains to be refined and verified by further studies.

Our Approaches Could Be Applied to Other Proteins Retaining Both Transactivation and Partner-Interacting Activities. Yeast two-hybrid analyses, in general, are very useful in studying protein/protein interactions (*9*). However, a number of important proteins, such as p53 (*28*, *29*) and human T-cell leukemia virus type-1 Tax protein (*30*, *31*), function in both transactivation and protein/protein interactions, and it is difficult to study their interactions with partner proteins using yeast two-hybrid systems because of their transactivation activities. The yeast one-hybrid-based random mutagenesis approach presented here could be applied to other proteins with both transactivation and protein-interacting abilities. To find mutants negative in transactivation and positive in protein/protein interactions, we first screened a random mutagenesis library for transactivation-negative mutants and then checked their abilities in binding to partners using yeast two-hybrid analyses. While screening a random mutagenesis library is time-consuming, a functional assay based on color development eases this library screening. In the case of SEI-1, after blue/white development, only 31 colonies (out of 5200 yeast colonies) were further evaluated using quantitative β-galactosidase activity assays, and only two of them were negative in transactivation, including the "perfect" mutant, RM29. Even if mutants totally negative in transactivation and positive in protein/protein interactions are not found, this approach can still identify those residues important for transactivation and then design multiple mutants that lose transactivation but retain protein/protein interaction abilities, such as the triple mutant L51A/L53H/L69A in the case of SEI-1.

Structure−Function Relationship of SEI-1. While the potential biological significance of SEI-1 is emerging, we are still at the very early stage of understanding the structure and function, as well as their relationship, of SEI-1. A combination of site-specific and random mutagenesis has allowed dissection of different activities and identification of segments and specific residues important for the specific function. A potential pitfall of such approaches is that the observed effects of the mutations can be caused by structural perturbations of the mutants. This problem is almost certain to cause some misinterpretations. However, this problem can be minimized by "cross examinations", studying the same problem with multiple approaches or using the same mutant to examine multiple properties. For example, the observation that the RM29 mutant (with five residues mutated) of SEI-1 retains full CDK4-binding activity provides a good assurance that this mutant is not structurally perturbed; therefore, its loss in the LexA-mediated transactivation can be confidently attributed to the mutated residues.

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